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## **PATENT ABSTRACTS OF JAPAN**

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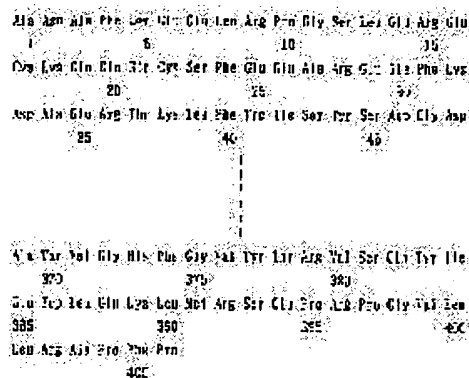
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**(54) MODIFIED BLOOD COAGULATION FACTOR VII**



(57)Abstract:

PROBLEM TO BE SOLVED: To obtain a new (activated) modified blood coagulation factor VII which has a modification at a specific site of an amino acid sequence, has an enhanced enzyme activity, and is useful as a medicine effective for treating a hemophilia inhibitor patient or the like.

SOLUTION: This is a new (activated) modified blood coagulation factor VII (FVII) which has at least one modification(s) selected from a group comprising cleaving the disulfide bond (159Cys-164Cys) consisting of 159th cysteine (159Cys) and 164th cysteine (164Cys) in blood coagulation factor VII, substituting, adding, or deleting at least a part of the amino acid sequence constituting the loop structure consisting of the amino acid sequence from 233rd threonine (233Thr) to 240th asparagine (240Asp) in FVII, and substituting, adding, or deleting at least a part of the amino acid sequence constituting the intervening sequence consisting from 304th arginine (304Arg) to 329th cysteine (329Cys) in FVII, and has an enhanced enzyme activity.

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## CLAIMS

[Claim(s)]

[Claim 1] The alteration field of the blood coagulation factor VII (following, FVII) characterized by including at least one alteration chosen from the following, or activated type blood coagulation factor VII (following, FVIIa).

(a) Cut the disulfide bond (159Cys-164Cys) which consists of the 159th cysteine (159Cys) in FVII, and the 164th cysteine (164Cys).

(b) Replace, add or delete the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd \*\*\*\*\* (233Thr) in FVII, or its part.

(c) Replace, add or delete the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part.

[Claim 2] The alteration field according to claim 1 characterized by cutting the concerned 159Cys-164Cys by replacing the above-mentioned 159Cys and 164Cys by amino acid residues other than Cys.

[Claim 3] The alteration field according to claim 1 or 2 which consists of an amino acid sequence of array table array number 4 publication.

[Claim 4] The alteration field according to claim 1 characterized by cutting 159Cys-164Cys and forming a disulfide bond (159Cys-299Cys) between 159Cys and 299Cys by amino acid residues other than Cys replacing 164Cys, and replacing the 299th valine (299Val) by Cys.

[Claim 5] The alteration field according to claim 1 or 4 which consists of an amino acid sequence of array table array number 6 publication.

[Claim 6] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence of 99-loop of FVII corresponds on the structure of other trypsin group serine proteases.

[Claim 7] The alteration field according to claim 1 or 6 other trypsin group serine proteases of whose are Homo-sapiens trypsins.

[Claim 8] The alteration field according to claim 1, 6, or 7 characterized by being replaced by Asp-Arg-Lys-Thr-Leu which has an amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) in the loop structure of corresponding on the structure of a Homo-

sapiens trypsin.

[Claim 9] The claim 1 which consists of an amino acid sequence of array table array number 8 publication, or the alteration field given in either 6-8.

[Claim 10] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part corresponds on the structure of other trypsin group serine proteases.

[Claim 11] The alteration field according to claim 1 or 10 other trypsin group serine proteases of whose are Homo-sapiens trypsins.

[Claim 12] The alteration field according to claim 1, 10, or 11 with which the amino acid sequence which constitutes the mediation amino acid sequence (170-loop may be called hereafter) of the 329th cysteine (329Cys) from the 310th cysteine (310Cys) in FVII, or its part is replaced, added or deleted.

[Claim 13] The claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys which has an amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) in the loop structure of corresponding on the structure of a Homo-sapiens trypsin, or the alteration field given in either 10-12.

[Claim 14] The claim 1 which consists of an amino acid sequence of array table array number 10 publication, or the alteration field given in either 10-13.

[Claim 15] The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of corresponding on the structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field according to claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of corresponding on the structure of a Homo-sapiens trypsin.

[Claim 16] The alteration field according to claim 1 or 15 which consists of an amino acid sequence of array table array number 12 publication.

[Claim 17] The drug constituent which contains the alteration field of a publication as an active principle in either of the claims 1-16.

[Claim 18] A medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent of a claim 17.

## DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

[0002]

[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [ itself ] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between bipartite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic

activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [ 4 / (4) / a thrombus induction of DIC, and ] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [ blood coagulation factor ] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [ the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less ] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product \*\*\*\*\* of FVII and the thing which enzyme activity went up in it are only [ one ], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

[0012]

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group – the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence ( [drawing 1](#) ). Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease ( [drawing 2](#) ). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed ( [drawing 3](#) ). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-\*\*) 159Cys from which 159Cys-164Cys combination was cut, and 164Cys by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-\*\*) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cys and 299Cys by amino acid residues other than Cys replacing 164Cys, and replacing the 299th \*\*\*\*\* (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd \*\*\*\*\* (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in [drawing 3](#) , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in [drawing 3](#) -- setting -- the serine protease of others [ FVII ] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example \*\*\*\*\* -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39)

replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [ a gene ] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

[0022]

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [ \*\*\*\* ] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [ as long as there is no notice especially the following ] transgenics etc. is TAKARA SHUZO, Toyobo, and par \*\*\*\*\* applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [ Molecular Basis ] of Thrombosis and cDNA array well-known at Hemostasis (– FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) – and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5 , and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared ( drawing 6 ). Moreover, about "pVII-6", the gene obtained in

drawing 5 using primer \*\* and \*\* of a publication is used as mold, and it was obtained by performing PCR further using primer \*\* and \*\*. Moreover, about "pVII-39", the gene obtained using primer \*\* and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array. [0026] The commercial \*\*\*\*\* cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (\*\*\*\*\* chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg /ml ] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [ as opposed to SDS-PAGE or commercial FVII for the purified alteration field ], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack \*\*\*\*, equivalent \*\*\*\*, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin; Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [ protein concentration (it measures by the Bradford method) ], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [ having freezing activity high two to 6 times ] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

| サンプル   | 変更内容                    | 凝固活性<br>U/ml | 蛋白濃度<br>μg/ml | 比活性<br>U/ml | 相対比<br>% |
|--------|-------------------------|--------------|---------------|-------------|----------|
| 血漿由来   | 天然品                     | 2,000        | 1,000         | 2,000       | 100      |
| VII-W  | 組換え野生型                  | 3,400        | 1,700         | 2,000       | 100      |
| VII-5  | 159Cys-164Cys の切断       | 4,954        | 1,032         | 4,800       | 240      |
| VII-6  | 159Cys-299Cys の形成       | 6,636        | 1,293         | 5,132       | 257      |
| VII-30 | loop99 を Trypsin 型へ     | 3,361        | 685           | 4,907       | 245      |
| VII-31 | loop170 を Trypsin 型へ    | 3,589        | 877           | 4,093       | 205      |
| VII-39 | loop99+170 を Trypsin 型へ | 8,954        | 773           | 11,584      | 579      |

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [ finishing / refining ] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible.

Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what \*\*, and showed hydration activity

higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

| 基質名           | 構造                 | 水解活性 / mOD <sub>405nm</sub> / min |         | 比<br>31/W |
|---------------|--------------------|-----------------------------------|---------|-----------|
|               |                    | VIIa-W                            | VIIa-31 |           |
| Chromozym tPA | D-Phe-Gly-Arg      | 37.6                              | 117.6   | 3         |
| S-2288        | H-D-Ile-Pro-Arg    | 25.8                              | 304.2   | 12        |
| S-2366        | pyro-Glu-Pro-Arg   | 11.5                              | 267.8   | 23        |
| S-2238        | H-D-Phe-Pip-Arg    | 11.3                              | 86.6    | 8         |
| Chromozym X   | D-Nile-Gly-Arg     | 13.0                              | 48.6    | 4         |
| S-2302        | H-D-Pro-Phe-Arg    | 7.4                               | 38.3    | 5         |
| S-2765        | Z-D-Arg-Gly-Arg    | 13.3                              | 20.6    | 2         |
| Chromozym TRY | CBz-Val-Gly-Arg    | 5.6                               | 28.2    | 5         |
| S-2444        | pyro-Glu-Gly-Arg   | 1.5                               | 18.9    | 13        |
| S-2222        | Bz-Ile-Glu-Gly-Arg | 5.6                               | 16.4    | 3         |
| S-2403        | pyro-Glu-Phe-Lys   | 0.3                               | 5.9     | 19        |

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

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 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC  
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## DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

[0002]

[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [ itself ] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiya et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between bipartite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique – respectively – (1) – more – high – about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [ 4 / (4) / a thrombus induction of DIC, and ] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [ blood coagulation factor ] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [ the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less ] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product \*\*\*\*\*s of FVII and the thing which enzyme activity went up in it are only [ one ], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

[0012]

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group – the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence ( drawing 1 ) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease ( drawing 2 ). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate



specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed ( drawing 3 ). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-\*\*) 159Cys from which 159Cys-164Cys combination was cut, and 164Cys by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-\*\*) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cys and 299Cys by amino acid residues other than Cys replacing 164Cys, and replacing the 299th \*\*\*\*\* (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd \*\*\*\*\* (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [ FVII ] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example \*\*\*\*\* -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [ a gene ] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by

refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

[0022]

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [ \*\*\*\* ] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [ as long as there is no notice especially the following ] transgenics etc. is TAKARA SHUZO, Toyobo, and par \*\*\*\*\* applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [ Molecular Basis ] of Thrombosa and cDNA array well-known at Hemostasis (– FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) – and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5, and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared ( drawing 6 ). Moreover, about "pVII-6", the gene obtained in drawing 5 using primer \*\* and \*\* of a publication is used as mold, and it was obtained by performing PCR further using primer \*\* and \*\*. Moreover, about "pVII-39", the gene obtained using primer \*\* and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.

[0026] The commercial \*\*\*\*\* cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>>

above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (\*\*\*\*\* chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [ /ml ] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [ as opposed to SDS-PAGE or commercial FVII for the purified alteration field ], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack \*\*\*\*, equivalent \*\*\*\*, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin; Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [ protein concentration (it measures by the Bradford method) ], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [ having freezing activity high two to 6 times ] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

| サンプル   | 変更内容                    | 凝固活性<br>U/ml | 蛋白濃度<br>μg/ml | 比活性<br>U/ml | 相対比<br>% |
|--------|-------------------------|--------------|---------------|-------------|----------|
| 血漿由来   | 天然品                     | 2,000        | 1,000         | 2,000       | 100      |
| VII-W  | 組換え野生型                  | 3,400        | 1,700         | 2,000       | 100      |
| VII-5  | 159Cys-164Cys の切断       | 4,954        | 1,032         | 4,800       | 240      |
| VII-6  | 159Cys-299Cys の形成       | 6,636        | 1,293         | 5,132       | 257      |
| VII-30 | loop99 を Trypsin 型へ     | 3,361        | 685           | 4,907       | 245      |
| VII-31 | loop170 を Trypsin 型へ    | 3,589        | 877           | 4,093       | 205      |
| VII-39 | loop99+170 を Trypsin 型へ | 8,954        | 773           | 11,584      | 579      |

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [ finishing / refining ] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microL, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible. Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what \*\*, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

| 基質名           | 構造                 | 水解活性/mOD <sub>405nm</sub> /min |         | 比<br>31/W |
|---------------|--------------------|--------------------------------|---------|-----------|
|               |                    | VIIa-W                         | VIIa-31 |           |
| Chromozym tPA | D-Phe-Gly-Arg      | 37.6                           | 117.6   | 3         |
| S-2288        | H-D-Ile-Pro-Arg    | 25.8                           | 304.2   | 12        |
| S-2366        | pyro-Glu-Pro-Arg   | 11.5                           | 267.8   | 23        |
| S-2238        | H-D-Phe-Pip-Arg    | 11.3                           | 86.6    | 8         |
| Chromozym X   | D-Nile-Gly-Arg     | 13.0                           | 48.6    | 4         |
| S-2302        | H-D-Pro-Phe-Arg    | 7.4                            | 38.3    | 5         |
| S-2765        | Z-D-Arg-Gly-Arg    | 13.3                           | 20.6    | 2         |
| Chromozym TRY | CBz-Val-Gly-Arg    | 5.6                            | 28.2    | 5         |
| S-2444        | pyro-Glu-Gly-Arg   | 1.5                            | 18.9    | 13        |
| S-2222        | Bz-Ile-Glu-Gly-Arg | 5.6                            | 16.4    | 3         |
| S-2403        | pyro-Glu-Phe-Lys   | 0.3                            | 5.9     | 19        |

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

SEQUENCE-LISTING<110> The-Chemo-Sero-Therapeutic Research-Institute<120> Recombinant-mutants of blood-coagulation factor VII<160> 12<210> 1<211> 1221<212> DNA<213> blood coagulation factor VII<400> 1GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC-TCC-CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 15 1015 TGC AAG GAGGAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATTTCT TAC AGT GAT GGG GAC 144AspAla Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser SerPro Cys Gln Asn Gly GlySer Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT AAC GAG AAC GGC288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACGGGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAAATA CCT ATT CTAGAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-TGG-AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC

1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC  
 ACG TGG TAC CTG ACG GGC ATC GTCAGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp  
 Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG  
 GTG TAC ACC AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg  
 Val Ser Gln Tyr Ile 370 375 380 GAG TGG CTG CAAAAGCTC ATG CGC TCA GAG CCA CGC  
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 Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
 Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn  
 Gly 85 90 95Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg  
 Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro  
 Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn AlaSerLys Pro Gln Gly Arg IleVal Gly Gly  
 Lys Val Cys Pro 145 150 155 160 Lys-Gly-Glu-Cys-Pro Trp Gln Val Leu Leu-Leu-Val-Asn-Gly-Ala-  
 Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser AlaAla180 185190 HisCys  
 PheAsp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200205Gly Glu His Asp Leu Ser Glu  
 His Asp Gly Asp Glu GlnSer Arg Arg 210 215 220Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly  
 Thr Thr Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245  
 250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe  
 Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu  
 Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys  
 Val Gly Asp Ser 305 310 315320 Pro AsnIleThr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
 325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly  
 Thr Trp Tyr Leu ThrGly IleVal Ser Trp Gly Gln Gly Cys 355 360 365AlaThr Val Gly His Phe Gly Val  
 Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro  
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 cDNA sequence coding thereof.<400> 3GCC AAC GCG TTC CTG-GAG-GAG-CTG CGG CCG  
 GGC TCC CTG GAGAGG GAG 48Ala Asn Ala PheLeu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg  
 Glu 1 5 10 15 TGC AAG GAG GAG CAG TGC TCCTTC GAG GAG GCC CGG GAG ATC TTC  
 AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG  
 GAG AGG ACG AAG CTG TTC TGGATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg  
 Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC  
 CAG AAT GGG GGC TCC TGC AAGGAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly  
 Ser Cys Lys Asp Gln 50 55 60CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG  
 GGC CGG AAC240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80  
 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys  
 Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95GGC TGT GAG CAG TAC  
 TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp  
 His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG  
 GCA GACGGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser  
 Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA  
 AAA AGA432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140  
 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC 480Asn  
 Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro 145 150 155 160 AAA GGG GAG  
 GCC CCA TGG CAG GTC CTG TTG TTGGTG AAT GGA GCT CAG 528Lys Gly Glu Ala Pro Trp  
 Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-  
 AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-  
 Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG AAC TGG AGGAAC  
 CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC  
 CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG  
 GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln  
 Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG  
 CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu  
 His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG  
 ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg

Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG  
 CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT  
 GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala  
 Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC  
 CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln  
 Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT  
 GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser  
 Asp Gly Ser 325 330 335 AAGGAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC  
 ACC CACTAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350  
 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104Arg  
 Gly Thr Trp Tyr-Leu-Thr-Gly-Ile Val Ser Trp Gly Gln-Gly-Cys 355 360 365 GCA-ACC-GTG-GGC-  
 CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152Ala Thr Val Gly His Phe Gly  
 Val Tyr Thr Arg Val-Ser-GlnTyr Ile 370 375 380 GAGTGG CTG CAA AAG CTC ATG CGC TCA  
 GAG CCA CGC CCA GGA GTC CTC1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro  
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 Pro CysGln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala  
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 GluGly TyrSer Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val GluTyr Pro Cys Gly  
 Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val GlyGly Lys Val  
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 175 Leu-Cys-Gly-Gly-Thr Leu Ile Asn Thr Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 His-Cys-Phe-  
 Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-ValLeu 195200 205Gly Glu HisAsp Leu Ser Glu His  
 Asp Gly Asp Glu Gln Ser Arg Arg 210 215220 ValAla Gln Val Ile Ile Pro Ser Thr TyrVal Pro Gly Thr  
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 Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu  
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 Val Gly Asp Ser 305310 315320 ProAsn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
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 GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe  
 Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTCTGG ATT TCT TAC AGT GAT GGG  
 GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT  
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 TGC CTC CCT GCC TTC GAG GGC CGGAAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala  
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 ATA CCTATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu  
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 Asn Thr Ile Trp Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-  
 TGG-AGG AAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys-Asn-Trp-Arg-Asn-Leu-  
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 215 220 GTG GCG CAG GTCATC ATC CCC AGC ACG TAC GTCCCG GGC ACC ACC AAC  
 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC  
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 CCC GAA CGG ACG TTC TCT GAGAGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr  
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 GTG GGC CAC TTT GGG GTG TAC-ACC-AGG-GTC-TCC CAG TAC ATC 1152Ala-Thr-Val-  
 Gly-His Phe Gly Val Tyr Thr-Arg-Val-Ser-Gln-Tyr-Ile 370 375 380 GAG-TGG-CTG-CAA-AAG  
 CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200Glu Trp Leu Gln Lys Leu Met Arg  
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 Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine  
 is replaced with Alanine and the 299Valine is replaced with Cysteine.<400> 6Ala Asn Ala Phe Leu Glu  
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 40 45 Gln Cys AlaSer Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile  
 Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His LysAsp Asp Gln Leu Ile  
 Cys Val Asn Glu Asn Gly 85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser  
 Cys 100 105 110 Arg Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro  
 Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130135 140Asn Ala Ser Lys Pro Gln  
 Gly Arg Ile ValGly Gly Lys Val Cys Pro 145 150 155 160Lys Gly Glu Ala Pro Trp Gln Val Leu Leu  
 Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala  
 Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195 200 205  
 Gly-Glu-His-Asp-Leu Ser Glu His Asp Gly-Asp-Glu-Gln-Ser-Arg-Arg 210215 220Val Ala Gln Vallie  
 Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235240 His AspIleAla Leu Leu Arg Leu His Gln  
 Pro Val ValLeu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg  
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 285AspArg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg 290 295 300 Leu Met Thr  
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 360 365Ala ThrVal Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu  
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 Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding  
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 AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys  
 Glu GluGln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG  
 CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile  
 Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAGAAT GGG GGC TCC  
 TGC AAG GAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55  
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 Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC

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CAC ACG GGC ACC AAGCGC TCCTGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr  
Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG  
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GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu  
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190 CAC TGT TTC GAC AAA ATCAA G-AAC-TGG-AGG-AAC-CTG ATC GCG GTG CTG  
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Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTGGCG CAG GTC ATC ATC  
CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr  
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CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr  
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CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala  
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GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG912Asp Arg Gly Ala Thr Ala Leu Glu  
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ACC AGGGTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln  
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GTC CTC 1200Glu Trp Leu Gln Lys Leu Met-Arg-Ser-Glu-Pro-Arg-Pro-Gly-Va-Leu 385 390 395  
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406<212> PRT <213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of  
blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th  
threonine are replaced with Asp-Arg-Lys-Thr-Leu.<400> 8Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro  
Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys LysGlu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys  
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His Lys Asp AspGln Leu Ile Cys Val Asn Glu Asn  
Gly 85 90 95Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg  
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro  
Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser LysPro Gln Gly Arg Ile ValGly Gly  
Lys Val Cys Pro 145 150 155 160 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala  
Gln 165 170 175 Leu Cys Gly Gly Thr Leu-Ile-Asn-Thr-Ile Trp Val Val Ser Ala-Ala 180 185 190 His-  
Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Va-Leu 195 200 205 Gly Glu His Asp Leu  
Ser Glu His Asp Gly AspGlu Gln Ser Arg Arg210215 220Val Ala Gln Val Ile Ile Pro Ser ThrTyr Asp  
Arg Lys Thr Leu Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr  
Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265270  
LeuAla Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr  
Ala Leu GluLeuMet Val LeuAsn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser  
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Pro Arg Pro Gly Val Leu 385 390 395 400Leu Arg Ala Pro Phe Pro 405 <210> 9 <211> 1206<212>  
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coagulation factor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding.[ thereof] <400>

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Ser-Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG CTG TTC TGG  
ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp  
Gly Asp 35 40 45 CAG TGT GCC TCA AGTCCA TGC CAGAAT GGG GGC TCC TGC AAG GAC  
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110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg  
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are replaced withGlu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 10Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro  
Gly Ser LeuGlu Arg Glu 1 5 10 15 Cys LysGlu Glu Gln Cys Ser Phe GluGlu Ala Arg Glu Ile Phe Lys  
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
Ala Phe Glu GlyArg Asn 65 70 75 80 Cys Glu Thr His LysAsp Asp Gln Leu Ile Cys Val Asn Glu Asn  
Gly 85 9095 Gly Cys Glu GlnTyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys  
His GluGly TyrSer Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125Pro Thr Val Glu Tyr Pro Cys  
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His Asp Gly-Asp-Glu-Gln-Ser-Arg-Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr ValPro

Gly Thr Thr Asn 225 230 235 240 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 Leu Ala  
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 Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro  
 Gly Lys Ile Thr Glu 305 310 315 320 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys  
 Lys 325 330 335 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345 350  
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 which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-  
 Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced  
 with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding.[ thereof] <400> 11 GCC AAC  
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 replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th

asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 12Ala Asn Ala Phe Leu Glu Glu  
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 Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395400 Pro

#### Field

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

#### Effect

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

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 Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC  
 ATG TGC CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn  
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 GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310  
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 TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC 1056Lys Asp Ser  
 Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC-ACG-TGG-TAC  
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 Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG GTG TAC ACC  
 AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr  
 Ile 370 375 380 GAG TGG CTG CAAAAGCTC ATG CGC TCA GAG CCA CGC CCA GGA GTC  
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 CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC 720Val Ala Gln Val  
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 CGC CTG CACCAGCCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln  
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 Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe  
 Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His Lys Asp AspGln Leu Ile Cys Val  
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 105 110 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 11 5 120 125 Pro-Thr-  
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 185 190His Cys Phe Asp Lys Ile LysAsn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205Gly Glu His  
 Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220Val Ala Gln Val Ile Ile Pro Ser  
 Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235 240His Asp Ile Ala LeuLeu Arg Leu His Gln Pro Val  
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 Ala Thr AlaLeu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu  
 Gln Gln Ser Arg Lys ValGly Asp Ser 305310 315320 ProAsn Ile Thr Glu TyrMet Phe Cys Ala Gly Tyr  
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 of-blood-coagulation factor-VII-in-which the 12 amino acid residues from the 311th leucine to 322th  
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Phe Cys Ala Gly Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys 325 330 335 GGG-GAC-AGT-GGA-  
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 GGC TGC GCA ACC GTGGGC CAC 1104Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val  
 Gly His 355 360 365 TTTGGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG  
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 His-Lys Asp Asp Gln Leu Ile-Cys-Val-Asn-Glu-Asn-Gly 85 90 95 Gly-Cys-Glu-Gln-Tyr Cys Ser Asp  
 His Thr-Gly-Thr-Lys-Arg-Ser-Cys 100 105 [110 ] Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly  
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 CysLeu Pro GluArg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe Val Arg Phe Ser Leu Val Ser  
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 acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid  
 residues from the 235thValine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leuand the 12  
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 amino acid residues from the 235th Valine to 239ththreonine are replaced with Asp-Arg-Lys-Thr-Leu  
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 Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180185 190 His Cys Phe Asp Lys Ile Lys  
 Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205GlyGlu His Asp Leu Ser Glu His Asp Gly Asp Glu  
 Gln Ser Arg Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225  
 230 235240 HisAsp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal  
 ProLeuCys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala PheVal Arg Phe Ser Leu  
 Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn  
 Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro-Gly-Lys-Ile-Thr Glu 305 310  
 315 320 Tyr-Met-Phe-Cys-Ala Gly Tyr Ser Asp Gly-Ser-Lys-Asp-Ser-Cys-Lys 325 330 335 Gly-Asp-  
 Ser Gly Gly Pro His Ala Thr HisTyrArg Gly Thr Trp Tyr 340 345350 LeuThr GlyIle Val Ser Trp Gly  
 Gln Gly Cys Ala Thr Val Gly His 355 360365Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu  
 TrpLeu Gln Lys 370 375 380Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385  
 390 395 400Pro

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#### TECHNICAL PROBLEM

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[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of  
 a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system  
 blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to  
 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K  
 dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to  
 in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation  
 blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called  
 hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-  
 153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a  
 piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).  
 [0003] If the enzyme activity of the FVIIa [ itself ] is very weak and it combines with the tissue factor

(TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between bipartite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [ 4 / (4) / a thrombus induction of DIC, and ] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [ blood coagulation factor ] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [ the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less ] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product \*\*\*\*\*s of FVII and the thing which enzyme activity went up in it are only [ one ], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

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## MEANS

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[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group – the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence ( [drawing 1](#) ) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease ( [drawing 2](#) ). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed ( [drawing 3](#) ). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-\*\*) 159Cys from which 159Cys-164Cys combination was cut, and 164Cys by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-\*\*) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cys and 299Cys by amino acid residues other than Cys replacing 164Cys, and replacing the 299th \*\*\*\*\* (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd \*\*\*\*\* (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in [drawing 3](#) , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in [drawing 3](#) -- setting -- the serine protease of others [ FVII ] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine

(310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example \*\*\*\*\* – for example, (b) and the combination of (c) – that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th \*\*\*\*\* (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [ a gene ] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

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## EXAMPLE

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[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [ \*\*\*\* ] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [ as long as there is no notice especially the following ] transgenics etc. is TAKARA SHUZO, Toyobo, and par \*\*\*\*\* applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [ Molecular Basis ] of Thrombosis and cDNA array well-known at Hemostasis (– FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) – and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-

W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> [view 4](#) of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, [drawing 4](#) is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in [drawing 5](#), and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared ([drawing 6](#)). Moreover, about "pVII-6", the gene obtained in [drawing 5](#) using primer \*\* and \*\* of a publication is used as mold, and it was obtained by performing PCR further using primer \*\* and \*\*. Moreover, about "pVII-39", the gene obtained using primer \*\* and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.

[0026] The commercial \*\*\*\*\* cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (\*\*\*\*\* chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium<sup>2+</sup>), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [ as opposed to SDS-PAGE or commercial FVII for the purified alteration field ], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack \*\*\*\*, equivalent \*\*\*\*, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin; Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [ protein concentration (it measures by the Bradford method) ], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [ having freezing activity high two to 6 times ] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

| サンプル   | 変更内容                      | 凝固活性<br>U/ml | 蛋白濃度<br>μg/ml | 比活性<br>U/ml | 相対比<br>% |
|--------|---------------------------|--------------|---------------|-------------|----------|
| 血漿由来   | 天然品                       | 2,000        | 1,000         | 2,000       | 100      |
| VII-W  | 組換え野生型                    | 3,400        | 1,700         | 2,000       | 100      |
| VII-5  | 159Cys-164Cys の切断         | 4,954        | 1,032         | 4,800       | 240      |
| VII-6  | 159Cys-299Cys の形成         | 6,636        | 1,293         | 5,132       | 257      |
| VII-30 | loop99 を Trypsin 型へ       | 3,361        | 685           | 4,907       | 245      |
| VII-31 | loop170 を Trypsin 型へ      | 3,589        | 877           | 4,093       | 205      |
| VII-39 | loop99 + 170 を Trypsin 型へ | 8,954        | 773           | 11,584      | 579      |

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium<sup>2+</sup>, Under the 37-degree C

condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [ finishing / refining ] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium<sup>2+</sup>, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible.

Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what \*\*, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

| 基質名           | 構造                 | 水解活性 / $\text{mOD}_{405\text{nm}}/\text{min}$ |         | 比<br>31/W |
|---------------|--------------------|---|---------|-----------|
|               |                    | VIIa-W  | VIIa-31 |           |
| Chromozym tPA | D-Phe-Gly-Arg      | 37.6  | 117.6   | 3         |
| S-2288        | H-D-Ile-Pro-Arg    | 25.8  | 304.2   | 12        |
| S-2366        | pyro-Glu-Pro-Arg   | 11.5  | 267.8   | 23        |
| S-2238        | H-D-Phe-Pip-Arg    | 11.3  | 86.6    | 8         |
| Chromozym X   | D-Nile-Gly-Arg     | 13.0  | 48.6    | 4         |
| S-2302        | H-D-Pro-Phe-Arg    | 7.4   | 38.3    | 5         |
| S-2765        | Z-D-Arg-Gly-Arg    | 13.3  | 20.6    | 2         |
| Chromozym TRY | CBz-Val-Gly-Arg    | 5.6   | 28.2    | 5         |
| S-2444        | pyro-Glu-Gly-Arg   | 1.5   | 18.9    | 13        |
| S-2222        | Bz-Ile-Glu-Gly-Arg | 5.6   | 16.4    | 3         |
| S-2403        | pyro-Glu-Phe-Lys   | 0.3   | 5.9     | 19        |

[0032]

## DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] Drawing showing the primary structure and the alteration site (asterisk) of FVII.

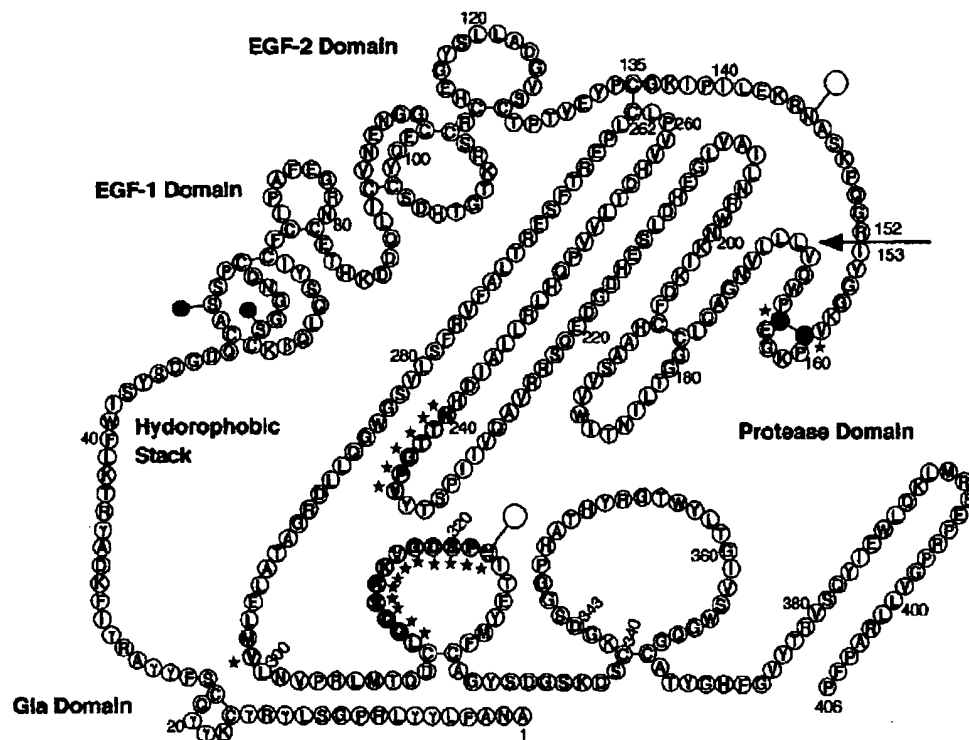
[Drawing 2] Drawing showing the basic structure of the serine protease on the basis of the protease domain amino acid sequence of FVII.

[Drawing 3] Drawing showing the 3D multi-alignment between the various trypsins group serine proteases of X-ray spacial-configuration known.

[Drawing 4] Drawing showing a part of amino acid sequence of wild-type FVII (FVII-Wild) and various FVII alteration fields. This view is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid sequence by the side of the end of N, neither performs an alteration, but it is the same than the 152nd arginine as that of a wild type.

[Drawing 5] Drawing showing the primer array for FVII alteration field production.

[Drawing 6] Drawing showing the construction technique of FVII alteration field manifestation vector.



Drawing 1

IVGGKVC PKGEC PWQV LLLVNGAQLCGGTLINTIWVVSAA  
 153                       $\beta$ ストランド1     $\beta$ ストランド2                       $\beta$ ストランド3 192  
 HCFDKIKNWRNLIAV LGEHDLSEHDGDEQSRRVAQVIIPS  
 193                       $\beta$ ストランド4                       $\beta$ ストランド5                      232  
 TYVPGTTNHDIALLR LHQP VVLT DHV VPLCLPERTFSERT  
 233                       $\beta$ ストランド6                      272  
 LAFVRFSLVSGWQQLDRGATAELMV LNVPRLMTQDCLQ  
 273                       $\beta$ ストランド7                       $\beta$ ストランド8                      312  
 QSRKVG DSPNITEYMECAGYS DGS KDSCKGDSGGPHATHY  
 313                       $\beta$ ストランド9                       $\beta$ ストランド10 352  
 RGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKL MR  
 353                       $\beta$ ストランド11                       $\beta$ ストランド12                      392  
 SEPRPGVLLRAPFP  
 393

Drawing 2



sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)  
sequence 2: ヒトトリプシン (pdb ID 1TRN)  
sequence 3: ブタ血液凝固第IX因子 (pdb ID 1PFY)  
sequence 4: ウシトリプシン (pdb ID 1TLD)  
sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)  
sequence 6: ヒトプロテインC (pdb ID 1AUT)  
sequence 7: ブタカリクレインA (pdb ID NPKA)  
sequence 8: ウシキモトリプシン (pdb ID 5CHA)  
sequence 9: ブタエラスターゼ (pdb ID 3EST)  
sequence10: ヒト $\alpha$ トロンビン (pdb ID 1PPB)  
sequence11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)  
sequence12: ラットトニン (pdb ID 1TON)  
sequence13: ヒト好中球エラスターゼ (pdb ID 1HNE)  
sequence14: ヒトウロキナーゼ型プラスミノーゲンアクチベータ (pdb ID 1LMW)  
sequence15: ヒトカテプシンG (pdb ID 1CGH)  
sequence16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)  
sequence17: ヒト組織型プラスミノーゲンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのC $\alpha$ 位置が1Å以内で保存されている構造保存部位を示す)

|             | $\beta$ ストランド5- $\beta$ ストランド6近傍の<br>アライメント | $\beta$ ストランド8- $\beta$ ストランド9近傍の<br>アライメント |
|-------------|---|---|
|             | @@@@@@@@:@@@:.....:@@@@@@@@@@@@             | @@@@@@@@@@@@:.....:@@@@@@@                  |
| sequence 1: | SRRVAQVIIPSTYVP---G-TTNHDIALLRLHQ           | ALELMVLNVPRMTQDCLQSRKVGDSNPITEYMFACG        |
| sequence 2: | FINAAKIIIRHPQYDR---K-TLNNDIMLIKLS           | PDELQCLDAPVLSQAKCEA-S-Y---PGKITSNMFCVG      |
| sequence 3: | RRNVIRAIIPHHSYNAT---VNKYSHDIALLELDE         | ATILQYLKVLVDRACTCL-R-ST-KFTIYSNMFCAG        |
| sequence 4: | FISASKSIVHPSYNS---N-TLNNDIMLIKLS            | PDVLKCLKAPILSDSSCKS-A-Y---PGQITSNMFCAG      |
| sequence 5: | VHEVEVVIKHNRFTE---E-TYDFDIIVLRKLT           | STRLEKMLEVPYVDRNSCKL-S-S---SFIITQNMFCAG     |
| sequence 6: | DLDEXEVFVHPNYSK---S-TTNNDIALLHLAQ           | TFVLNFIKIPVPHNECSE-V-M---SNMVSSEMLCAG       |
| sequence 7: | FFGVTADEFHPGFNLSA-DGKDYSHDLMLRLQS           | PDEIQCVQLTLLQNTFCA-D-AH-PDEVTESEMLCAG       |
| sequence 8: | KLKIAKVFKNKYNS---L-TINNDITLKLST             | PDRLLQASLPLLSNTNCKK-Y-W---GTKIKDAMICAG      |
| sequence 9: | YVGQKIVVHPYWN---D-DVAAGYDIALLRLAQ           | AQTLQQAAYLPTVDYAISS-SSYW-GSTVKNSMVCAC       |
| sequence10: | ISMLEKIYIIPRYNW---RENLDRIALMKLKK            | PSVLQVVNLPIVERPVCKD-S-T---RIRITDNMFCAG      |
| sequence11: | HFSVAQVFLN-KYDA---E-NKLNIDILLQLSS           | AQVLQELAVTVVT-FFC-----R-PHNICTF             |
| sequence12: | RRLVRQSFRHPDYIP-LPVHDHSNDLMLHLSE            | SHDLQCVNIHLISNEKCI-E-TY-KDNVTDVMLCAG        |
| sequence13: | VFAVQRIKED-GYDP---V-NLLNDIVILQLNG           | ASVLQELAVTVVT-SLC-----R-RSNVCTL             |
| sequence14: | KFEVENLILHKDYSA-D-TLAHNDIALLKIRS            | PEQLKMTVVKLISHRECQPH-YY-GSEVTKMLCAA         |
| sequence15: | HITARRAIRHPQYNQ---R-TIQNDIMLLQLSR           | TDTLREVQLRVQRDRQCLR-I-F---GSYDPRRQICVG      |
| sequence16: | KIKVEKQIHESYNS---V-PMIJDIMLLKLEK            | SYTLREVELRIMDEKACVD-Y-R---Y-YEYKFOVCVG      |
| sequence17: | KFEVEKYIVHKEFDD---D-TYNDIALLQLKS            | SERLKEAHVRLYPSSRCTSQH-LL-NRTVTDNMLCAG       |

Drawing 3

VII-Wild

IVGGKVC PKGEC PWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDSPNITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

VII-5

IVGGKVC APKGEAPWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDSPNITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

VII-6

IVGGKVC PKGEAPWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMCLNVPRLMTQDCLQQRKVGDSPNITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

VII-30

IVGGKVC PKGEC PWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYDRKTLNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDSPNITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

VII-31

IVGGKVC PKGEC PWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

VII-39

IVGGKVC PKGEC PWQV LLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD  
LSEHDGDEQSRRVAQV IIPSTYDRRTLNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMF CAGY  
SDGSKDSCKGDSGGPHATHYRG TWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

(下線部は改変部位を表す)

①VII-PWN Sense ; 5'-GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'

Factor VII Wild type のシグナル配列からのプライマーデザイン

5'-GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'

SalI M V S Q A L R L L C L L

②VII-PWC AntiS ; 5'-CCCGGATCCCTAGGGAATGGGGCTCGCAGGAGGACTCCTGGGCG-3'

Factor VII Wild type のカルボキシ末端までのプライマーデザイン

5'-CCCGGATCCCTAGGGAATGGGGCTCGCAGGAGGACTCCTGGGCG-3'

BamHI

③VII-P5-1 Sense ; 5'-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3'

④VII-P5-2 AntiS ; 5'-GACCTGCCATGGGGCCTCCCCCTTGGGGGCCACCTTGCCCCCACAAT-3'

VII-5のプライマーデザイン (C159A, C164A)

5'-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3'

3'-TAACACCCCCCGTTCCACCGGGGGTTCCCTCCGGGGTACCGTCCAG-5'

I V G G K V A P K G E A P W Q V

⑤VII-P6-1 Sense ; 5'-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3'

⑥VII-P6-2 AntiS ; 5'-GACCTGCCATGGGGCCTCCCCCTTGGGGCA-3'

VII-6のプライマーデザイン① (C164A)

5'-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3'

3'-ACGGGGTTTCCCTCCGGGGTACCGTCCAG-5'

C P K G E A P W Q V

⑦VII-P6-3 Sense ; 5'-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3'

⑧VII-P6-4 AntiS ; 5'-CCGGGGCACGTTGAGGCACATGAGTCCAG-3'

VII-6のプライマーデザイン② (V299C)

5'-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3'

3'-GACCTCGAGTACACGAGTTGCACGGGGCC-5'

L E L M C L N V P R

⑨VII-P30-1 Sense ; 5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3'

⑩VII-P30-2 AntiS ; 5'-CAGCGCGATGTCGTGGTTACAGAGTCTTCTGTCTGCTACGTCCTGGGGAT-3'

VII-30のプライマーデザイン (VPGTTN→DRKTLN)

5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3'

3'-TAGGGGTCGTGCATGCTGTCTTCTGAGACTTGGTGCTGTAGCGCGAC-5'

I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense ; 5'-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCAGGAGTACATG-3'

⑫VII-P31-2 AntiS ; 5'-CATGTACTCCGTGATCTTCCAGGGTAGGAGGCTTCGCAGTCTTGGGTAT-3'

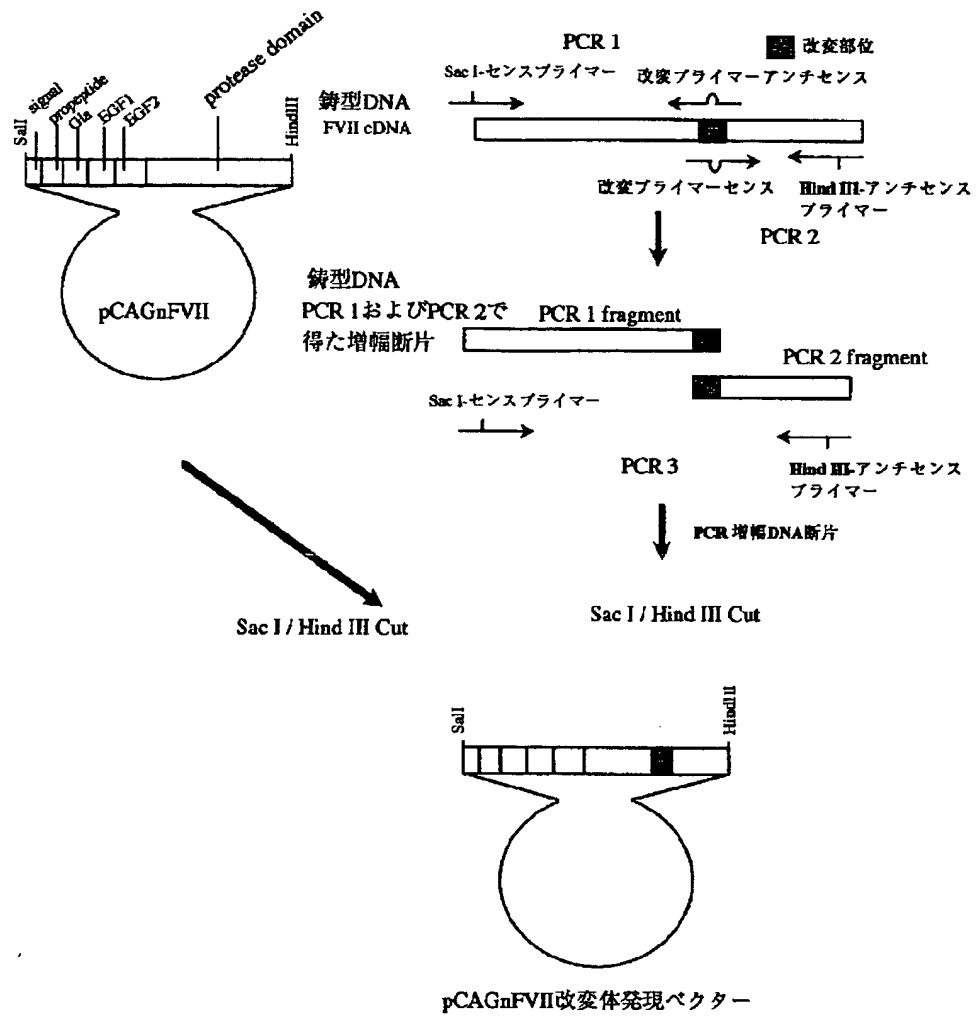
VII-31のプライマーデザイン (LQSRKVGDSFN→EASYPGR)

5'-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCAGGAGTACATG-3'

3'-TACTGGGTCTGACGCTTCGGAGGATGGGACCTTCTAGTGCCTCATGTAC-5'

M T Q D C E A S Y P G K I T E Y M

Drawing 5



Drawing 6

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(54) 【発明の名称】 血液凝固第V I I 因子改変体

(57) 【要約】

【課題】 酵素活性を増強させた血液凝固第  
V I I 因子 (以下、F V I I) 及び/または活性化型血  
液凝固第V I I 因子 (以下、F V I I a) の改変体を作  
製する。

【解決手段】 下記から選択される少なくとも一  
つの改変を含むことを特徴とするF V I I / F V I I a  
改変体及び当該改変体を有効成分として含有する医薬品  
組成物。(a) F V I I の159C y s-164C y s の切  
断、(b) F V I I 内の、233番目のスレオニン (23  
3T h r) から240番目のアスパラギン (240A s n)  
のアミノ酸配列からなるループ構造を構成するアミノ酸  
配列またはその一部を、置換、追加または削除、(c)  
F V I I 内の、304番目のアルギニン (304A r g)  
から329番目のシステイン (329C y s) の介在アミ  
ノ酸配列を構成するアミノ酸配列またはその一部を、置  
換、追加または削除。

**【特許請求の範囲】**

【請求項1】 下記から選択される少なくとも一つの改変を含むことを特徴とする血液凝固第ⅤⅡ因子（以下、FⅤⅡ）または活性化型血液凝固第ⅤⅡ因子（以下、FⅤⅡa）の改変体。

（a）FⅤⅡ内の、159番目のシステイン（159Cys）と164番目のシステイン（164Cys）からなるジスルフィド結合（159Cys-164Cys）を切断する。

（b）FⅤⅡ内の、233番目のスレオニン（233Thr）から240番目のアスパラギン（240Asn）のアミノ酸配列からなるループ構造（以下、99-loopと称することもある）を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

（c）FⅤⅡ内の、304番目のアルギニン（304Arg）から329番目のシステイン（329Cys）の介在アミノ酸配列を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

【請求項2】 上記159Cysと164CysをCys以外のアミノ酸残基によって置換することにより、当該159Cys-164Cysが切断されることを特徴とする請求項1に記載の改変体。

【請求項3】 配列表配列番号4記載のアミノ酸配列からなる請求項1または2に記載の改変体。

【請求項4】 164CysをCys以外のアミノ酸残基によって置換し、かつ、299番目のバリン（299Val）をCysに置換することにより、159Cys-164Cysが切断され、かつ159Cysと299Cys間においてジスルフィド結合（159Cys-299Cys）が形成されることを特徴とする請求項1に記載の改変体。

【請求項5】 配列表配列番号6記載のアミノ酸配列からなる請求項1または4に記載の改変体。

【請求項6】 FⅤⅡの99-loopのアミノ酸配列が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項1に記載の改変体。

【請求項7】 他のトリプシン族セリンプロテアーゼがヒトリプシンである請求項1または6に記載の改変体。

【請求項8】 FⅤⅡの99-loop内の235番目のバリン（235Val）から239番目のスレオニン（239Thr）までのアミノ酸配列が、ヒトリプシンの構造上対応するループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換されることを特徴とする請求項1、6または7に記載の改変体。

【請求項9】 配列表配列番号8記載のアミノ酸配列からなる請求項1または6から8のいずれかに記載の改変体。

【請求項10】 FⅤⅡ内の、304番目のアルギニン（304Arg）から329番目のシステイン（329Cys）の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項1に記載の改変体。

【請求項11】 他のトリプシン族セリンプロテアーゼがヒトリプシンである請求項1または10に記載の改変体。

【請求項12】 FⅤⅡ内の、310番目のシステイン（310Cys）から329番目のシステイン（329Cys）の介在アミノ酸配列（以下、170-loopと称することもある）を構成するアミノ酸配列またはその一部が、置換、追加または削除される請求項1、10または11に記載の改変体。

【請求項13】 FⅤⅡの170-loop内の311番目のロイシン（311Leu）から322番目のアスパラギン（322Asn）までのアミノ酸配列が、ヒトリプシンの構造上対応するループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換されることを特徴とする請求項1または10から12のいずれかに記載の改変体。

【請求項14】 配列表配列番号10記載のアミノ酸配列からなる請求項1または10から13のいずれかに記載の改変体。

【請求項15】 FⅤⅡの99-loop内の235番目のバリン（235Val）から239番目のスレオニン（239Thr）までのアミノ酸配列が、ヒトリプシンの構造上対応するループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換され、かつ、170-loop内の311番目のロイシン（311Leu）から322番目のアスパラギン（322Asn）までのアミノ酸配列が、ヒトリプシンの構造上対応するループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換されることを特徴とする請求項1に記載の改変体。

【請求項16】 配列表配列番号12記載のアミノ酸配列からなる請求項1または15に記載の改変体。

【請求項17】 請求項1から16のいずれかに記載の改変体を有効成分として含有する医薬品組成物。

【請求項18】 請求項17の医薬品組成物からなる血友病インヒビター患者の治療に有効な薬剤。

**【発明の詳細な説明】****【0001】**

【発明の属する技術分野】 本願発明は、酵素活性を増強させた血液凝固第ⅤⅡ因子（以下、FⅤⅡと称することがある）及び／または活性化血液凝固第ⅤⅡ因子（以下、FⅤⅡaと称することがある）の改変体に関するものである。詳細には、本願発明は、FⅤⅡに特有なアミノ酸配列を、置換・欠損することにより、活性が増強されたFⅤⅡ/FⅤⅡa改変体、当該改変体を有効成分として含有する医薬品組成物、及び当該医薬

品組成物からなる血友病インヒビター患者の治療に有効な薬剤に関するものである。

#### 【0002】

【従来の技術および解決すべき課題】FVⅡはビタミンK依存性の血液凝固因子であり、外因系血液凝固の開始因子であることは広く知られている。他のビタミンK依存性凝固因子と同様にN末端から35残基までのアミノ酸配列に10個のγカルボキシグルタミン酸（以下、GⅠaと称することがある）からなるGⅠa領域を有している（Proc. Natl. Acad. Sci. USA, vol. 83, p. 2412-2416, 1986）。FVⅡは、*in vitro*において、活性化血液凝固第X因子（以下、FXaと称することがある）、活性化血液凝固第ⅠX因子（以下、FⅠXaと称することがある）またはトロンビン（以下、FⅠⅡaと称することがある）によって、152Arg-153Ileが加水分解され、一つのS-S結合で架橋されたH鎖とL鎖から構成される活性型FVⅡすなわちFVⅡaに変換されることは知られている（J. Biol. Chem., vol. 251, p. 4797-4802, 1976）。

【0003】FVⅡa自体の酵素活性は極めて弱く、補酵素である組織因子（TF）と結合すると劇的に上昇する（Komiya et al., Biochemistry, 29(40), pp. 9418-25(1990)）。FVⅡaとTFの1次構造、その複合体の結晶構造、さらに両分子間の結合部位もアミノ酸残基レベルで判明しているが、その触媒活性増幅機構の詳細（TF結合に伴う立体構造変化）は依然として不明である（Banner et al., et al., Nature 380(6569): pp. 41-6(1996)）。

【0004】血友病A及び血友病B患者に対する補充療法として、血液凝固第Ⅷ因子（以下、FⅧと称することがある）及び血液凝固第ⅠX因子（以下、FⅠXと称することがある）製剤の投与が行なわれている。しかし、当該治療法に伴いFⅧ及びFⅠXに対する中和抗体（インヒビターと呼ばれることもある）の出現が問題視されている。

【0005】このようなインヒビターを生じた血友病患者の対処療法として、(1)FⅧ因子の過剰投与、(2)ブタFⅧ因子の投与、(3)FⅠⅠ、FVⅡ、FⅠX及びFXからなる複合体製剤の投与、(4)FVⅡa製剤の投与などがある。しかしながらこれらの方法は、それぞれ(1)については、より高価なインヒビターの誘導による症状悪化、(2)については、抗原性によるショック、(3)については、血栓・DICの誘発、(4)については、治療効果が不十分であることや大量・頻回投与によりコストが高いなどの問題を抱えている。これらの中で、効果と危険性のバランスを考慮した場合、最も効率的なものは(4)のFVⅡa製剤の投与である。しかしながら、FVⅡa製剤はその活性の弱さのため、止血効果を発揮するには、前述したように大量投与と頻回投

与を必要とし、治療コストを大きく高めている。また、その治療効果も血友病患者に対して行われている従来の補充療法に比べれば充分とはいえない。

【0006】この問題を解決するための手段として、酵素活性を上昇させたFVⅡの改変体を作製することが挙げられるが、これは一般的に困難であることが知られている（タンパク質の構造入門、勝部幸輝ら監修、教育社発行、1992年）。特に、血液凝固因子について、以下の理由により改変による活性増強は困難と考えられている。

【0007】血友病は血液凝固因子の異常であるが、量的欠損に伴う活性低下と質的異常による活性低下の2つに分類される。このうち質的異常の多くは（ポイント）ミューテーションであることが知られており、FⅠXの異常である血友病Bの患者の解析が行われた結果、FⅠXの構造全域にわたって分子異常が存在することが明らかとなり、中にはたった1個のアミノ酸が置換されただけで、活性が1%以下になる例が多数ある。従って、血液凝固因子についてむやみに改変を行っても、活性低下を招くのは明らかである。

【0008】また、Alanine Scanningで得られた情報（Dickinson et al., Proc. Natl. Acad. Sci. USA, 93(25), pp. 14379-84(1996)）によれば、FVⅡの112個のAlanine置換体について、その中で酵素活性が上がったものは唯一1つであり、しかもその規則性は見いだされていない。

【0009】その他の試みとして、Hofnerらは、FⅠXを構成する一部のドメインの数アミノ酸残基から構成される構造単位を欠損・置換する方法を用い、合成基質活性を上昇させたFⅠXフラグメント改変体を作製した（EMBO J, 16(22), pp. 6626-35(1997)）。しかしながら、これはインタクトなFⅠXではなく、FⅠXの部分フラグメントを大腸菌で発現させたもので合成基質活性を見ているに過ぎないため、血液凝固活性を増強しうどころか血液凝固活性すら有さないものである。さらに、これはFⅠXに関するものであり、構造も特異性も全く異なる別物質であるFVⅡに対して何ら示唆するものではなく、FVⅡの酵素活性を増強させた改変体についても、これまで何ら報告はない。

【0010】このように、強い酵素活性を有する改変体の作製は、特に血液凝固因子においては困難と考えられていた。FⅠXにおいて、その部分フラグメントについて合成基質活性を上げる試みはなされたものの、インタクトな分子として高い酵素活性を有する血液凝固因子の改変体についてはこれまでも報告例はない。

【0011】従って、本発明の解決すべき課題は、一般に血液凝固因子の改変は困難と考えられている状況において、血友病インヒビター患者の治療に有効な強い活性を有するFVⅡ及び／またはFVⅡaを作製・提供することである。

## 【0012】

【課題を解決するための手段】上記のような状況において、本発明者らは、それ自身高い酵素活性を有するFVIIを作製すべく鋭意研究を重ね種々の検討を行った結果、本願発明を完成するに至った。本願発明は、FVIIと各種セリンプロテアーゼとアミノ酸配列構造を比較し、FVIIに特有のアミノ酸配列部位を明確にし、その特有な部位を、欠損・置換することにより、活性が増強されたFVII及び／またはFVIIa改変体を作製することに成功したものである。

## 【0013】

【発明の構成】トリプシン族に類する一群のセリンプロテアーゼの基本構造は、約250残基からなり、アミノ酸配列上でおおよそ、その前半と後半の2つのドメインに分けられる(図1)。各ドメイン内にはそれぞれ6本のβストランドがあり、プロテアーゼとして計12本のβストランドを有する構造で形成されている(図2)。これら12本のβストランドはいわばセリンプロテアーゼの骨格構造となっており、各ストランド間をつなぐループないし、ヘリックス領域が、その基質特異性やコファクターとの反応性などのプロテアーゼ活性を担っていると考えられている。セリンプロテアーゼの例としては、FII、FVII、FVIII、FIX、FX等の各種血液凝固因子、プラスミン等の血栓溶解酵素、またはトリプシン、キモトリプシン、エラスターゼなどの消化酵素がある。そこで、FVIIをはじめとする各種セリンプロテアーゼのアミノ酸配列構造の比較を行い、FVIIに特徴的な領域を特定した(図3)。そして、これらの部位を改変のターゲットとし、他のセリンプロテアーゼの構造を参考に、FVIIのアミノ酸配列を欠損・置換することによって、高い酵素活性を有するFVII改変体を作製した。これらの改変体について詳細に説明する。

【0014】(a) 159Cys-164Cys結合が切断された改変体

(a-①) 159Cysと164CysをCys以外のアミノ酸残基によって置換することにより、当該159Cys-164Cysが切断された改変体(VII-5)。この改変体の具体例として、Cysをそれぞれアラニン(Ala)に置換したものを配列表配列番号3または4に記載した。ここで、置換に用いるCys以外のアミノ酸残基の一例として、Alaを選択したが、置換によって、Cys-Cys結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、任意のアミノ酸が選択可能である。

【0015】(a-②) 164CysをCys以外のアミノ酸残基によって置換し、かつ、299番目のヴァレイン(299Val)をCysに置換することにより、159Cys-164Cysが切断され、かつ159Cysと299Cys間においてジスルフィド結合(159Cys-299Cys)

が形成された改変体(VII-6)。この改変体の具体例として、Cys以外のアミノ酸残基としてAlaを用いて置換したものを配列表配列番号5または6に記載した。ここで、置換に用いるCys以外のアミノ酸残基については上述の通り、置換によって159Cys-164Cys結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、Ala以外の他のアミノ酸が選択可能である。

【0016】(b) FVII内の、233番目のスレオニン(233Thr)から240番目のアスパラギン(240Asn)のアミノ酸配列からなるループ構造(以下、99-loopと称することもある)を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。

この領域は、図3に示すようにセリンプロテアーゼに共通に存在するβストランド5とβストランド6の間に介在するアミノ酸配列を含むものである。この領域を他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。さらに、具体的な例として、FVIIの99-loop内の235番目のバリン(235Val)から239番目のスレオニン(239Thr)までのアミノ酸配列が、トリプシンのループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換された改変体(VII-30)が挙げられる。この改変体を配列表配列番号7または8に記載した。

【0017】(c) FVII内の、304番目のアルギニン(304Arg)から329番目のシステイン(329Cys)の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。特にこの領域は、図3に示すように、セリンプロテアーゼに共通に存在するβストランド8とβストランド9の間に介在するアミノ酸配列において、FVIIは他のセリンプロテアーゼと比較して数アミノ酸残基長いという特徴を有することから、FVII改変における好適なターゲットとなりうるものと推測される。この領域を、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。また、FVII内の置換、追加、削除しうる好ましい領域は、310番目のシステイン(310Cys)から329番目のシステイン(329Cys)のアミノ酸配列からなるループ構造(170-loopと称することもある)を構成するアミノ酸配列またはその一部である。さらに、具体的な例として、FVIIの170-loop内の311番目のロイシン(311Leu)から322番目のアスパラギン(322Asn)までのアミノ酸配列が、ヒトトリプシンのループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Ly



sで置換された改変体(VII-31)が挙げられる。この改変体を配列表配列番号9または10に記載した。

【0018】さらに、上記(a)から(c)の改変を適宜組み合わせることも可能である。その具体例として、例えば、(b)と(c)の組み合わせ、すなわち、FVIIの99-loop内の235番目のバリン(235Val)から239番目のスレオニン(239Thr)までのアミノ酸配列が、ヒトリプシンのループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換され、かつ、170-loop内の311番目のロイシン(311Leu)から322番目のアスパラギン(322Asn)までのアミノ酸配列が、トリプシンのループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換された改変体(VII-39)が挙げられる。この改変体を配列表配列番号11または12に記載した。

【0019】上述した改変体は、遺伝子組換え法を用いて得ることができる。発現宿主としては、動物細胞等の真核細胞が好ましい。本発明の改変体は、上記各改変体のアミノ酸配列をコードするcDNAを適当な発現ベクターに組み込み、宿主細胞にトランスフェクトし、目的の遺伝子を発現している細胞をクローニングし、得られた安定発現株を培養後、精製することにより得られる。

【0020】本願発明のFVII改変体は各種化学処理等を行い、活性化型FVII(FVIIa)改変体として使用することができる。

【0021】本願発明のFVII/FVIIa改変体は、治療、診断または他の用途のために製薬学的調合剤に処方することができる。静脈内投与のための調合剤に対しては、組成物を、通常、生理学的に適合しうる物質、例えば塩化ナトリウム、グリシン等を含み、かつ生理学的条件に適合しうる緩衝されたpHを有する水溶液中に溶解する。また、長期安定性の確保の観点から、最終的剤型として凍結乾燥剤の形態をとることも考慮されうる。なお、静脈内に投与される組成物のガイドラインは政府の規則、例えば「生物学的製剤基準」によって確立されている。本願発明のFVII/FVIIa改変体からなる医薬品組成物の具体的な用途としては、FVIIまたはFIXの補充療法により当該血液凝固因子に対してインヒビターを生じた血友病インヒビター患者の治療が挙げられる。

#### 【0022】

【実施例】本願発明を実施例により例示するが、これら実施例は本願発明を限定するものではない。本願発明について添付図面を参照して特定の実施例にて例示する。実施例は改変体を動物細胞(CHO-K1)の培養上清中に発現させたものである。以下特に断りが無い限り、遺伝子組換えに関わる試薬等は、宝酒造、東洋紡、パーキンエルマーアプライドNew England Biolabs社の製品を用いた。

【0023】《実施例1. FVIIcDNAのクローニング》ヒト肝臓cDNAライブラリー(宝酒造)を購入し、文献等(Molecular Basis of Thrombosis and Hemostasis)で公知のcDNA配列(配列表配列番号1に記載)を基にSalIサイトを付加したFVII合成DNAセンスプライマー(VII-PWN; GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTCTGCCTTCTG)及び、BamHIサイトを付加したアンチセンスプライマー(VII-PWC; CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG)を用いてPCRを行い、市販のクローニングベクターpCRII(Invitrogen社)にクローニングした。この際、常法によりDNAシークエンスを行い、文献等で公知の配列(Hagen FS et al, PNAS 1986; 83; 2412-6)を有することを確認した。

【0024】《実施例2. FVII発現ベクターの調製》発現ベクターpCAGn(特許第2824434号公報)をSalI、BamHIで消化し、そこにFVIIをコードした配列を含む上記実施例1で調製したDNAフラグメントをSalI、BamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のLB寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晚培養し、目的の発現プラスミドを抽出精製し「pVII-W」を調製した。この発現ベクターのDNAシークエンスを行い、目的の遺伝子配列を有することを確認した。

【0025】《実施例3. 改変体発現ベクターの調製》図4に示すアミノ酸配列を有する各FVII改変体を、以下の方法で作成した。なお図4は、FVIIの153番目のイソロイシンよりC末側のアミノ酸配列のみ示したもので、152番目のアルギニンよりN末側のアミノ酸については、いずれも改変は行っており野生型と同じである。図5に示す合成DNAプライマーを用いてFVII遺伝子を鋳型としてPCRを行いそれぞれの増幅断片を得る。各増幅断片と、発現ベクターpCAGnをSalI及びBamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のLB寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晚培養し、目的の発現プラスミドを抽出精製し「pVII-5」、「pVII-30」、及び「pVII-31」を調製した(図6)。また、「pVII-6」については、図5に記載のプライマー⑤及び⑥を用いて得られた遺伝子を鋳型にし、さらにプライマー⑦及び⑧を用いてPCRを行うことにより得られた。また、「pVII-39」については、プライマー⑨及び⑩を用いて得られた遺伝子を鋳型にし、さらにプライマー⑪及び⑫を用いてPCRを行うことにより得られた。さらにDNAシークエンスを行い、これらのプラスミドが目的の配列を有することを確認した。

【0026】《実施例4. 各改変体の培養上清への発現及び精製》上記発現ベクターを、市販のリポフェクシン

試薬でCHO細胞に対して形質導入を行い、G418(1mg/ml)で選択し、目的の遺伝子を発現している細胞を限外希釈法によりクローニングした。FVII改変体の発現の確認は、市販のFVIIに対するELISAキット(アセラクロムFVII; Diagnostica Strago社)で行った。得られた安定発現株を無血清培地(ASF104、味の素、ペニシリン、ストレプトマイシン、20μg/mlビタミンK、1mM酪酸)で培養し、抗ヒトFVIIモノクローナル抗体カラムで精製した(特許第2824430号公報)。平衡化・洗浄及び溶出は、平衡化・洗浄バッファー(50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidinium-HCl, 2mM  $Ca^{2+}$ )、溶出バッファー(50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidinium-HCl, 10mM EDTA)を用いて行った。純化された改変体をSDS-PAGE、または市販のFVIIに対する抗体を用いて、ウェスタンブロットを行い、FVII改変体であることを確認し

た。

【0027】《実施例5. 各改変体の凝固活性の測定》各改変体の凝固活性は常法に従い、FVII欠乏血漿を用いた凝固法で測定した。精製した各改変体を50~5ng/mlになるようにTris-BSAで希釈し、FVII欠乏血症と等量混ぜ、37℃で3分加温後、再脂質化TF(トロンボプラスチン; Dade社)を等量添加し、凝固反応を開始させた。凝固時間を測定し、標準曲線と希釈率より凝固活性を求めた。凝固活性を蛋白濃度(Bradford法で測定)当たりに換算し比活性を求めた結果を表1に記す。その結果、本発明のFVII改変体は、血漿由来FVII及び野生型組換えFVIIと比較して、2~6倍高い凝固活性を有することが明らかとなった。

【0028】

【表1】

| サンプル   | 改変内容                 | 凝固活性<br>U/ml | 蛋白濃度<br>μg/ml | 比活性<br>U/ml | 相対比<br>% |
|--------|----------------------|--------------|---------------|-------------|----------|
| 血漿由来   | 天然品                  | 2,000        | 1,000         | 2,000       | 100      |
| VII-W  | 組換え野生型               | 3,400        | 1,700         | 2,000       | 100      |
| VII-5  | 159Cys-164Cysの切断     | 4,954        | 1,032         | 4,800       | 240      |
| VII-6  | 159Cys-299Cysの形成     | 6,636        | 1,293         | 5,132       | 257      |
| VII-30 | loop99をTrypsin型へ     | 3,361        | 685           | 4,907       | 245      |
| VII-31 | loop170をTrypsin型へ    | 3,589        | 877           | 4,093       | 205      |
| VII-39 | loop99+170をTrypsin型へ | 8,954        | 773           | 11,584      | 579      |

【0029】《実施例6. 活性化された各改変体の調製》精製した各改変体を、50mM Tris, pH 7.45, 0.1M NaClに透析し、FXaを1/100(モル比)に加え、50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100μg/mlリン脂質(Platerin(登録商標) Organotecnica社)、10mM  $Ca^{2+}$ 、37℃の条件下、1~60分でインキュベーションし活性化した。活性化後、50mM Benzamidinium-HClを加えて反応を停止し、抗ヒトFVIIモノクローナル抗体カラムで精製した(実施例4と同じ方法)。精製済みの各活性化改変体はTBS pH 8.0(0.1% PEG 8000含有)に透析し、-80℃に凍結保存した。活性化の程度は、SDS-PAGEで確認した。

【0030】《実施例7. 活性化された各改変体の合成基質に対する水解活性測定》実施例6に従い活性化され

た改変体VIIa-31を0.1μMになるまで50mM Tris-HCl, 100mM NaCl, 10mM  $Ca^{2+}$ 、0.1% PEG 8000, pH 8.0で希釈し、そこに種々の合成基質を最終濃度1.0mMになるように加え、最終容量を200μlとし、30℃で反応させ、1分間当たりの基質の水解量を見た。温度制御が可能なmicroplate reader Spectra max plus(Molecular device社)でpNAの遊離を405nmによる発色度として測定した。この結果を表2に示す。本発明の改変体の一つであるVIIa-31は、何れの合成基質に対しても野生型(VIIa-W)より高い水解活性を示し、その範囲は2~23倍であった。

【0031】

【表2】

| 基質名           | 構造                 | 水解活性 / mOD <sub>405nm</sub> /min |         | 比<br>31/W |
|---------------|--------------------|----------------------------------|---------|-----------|
|               |                    | VIIa-W                           | VIIa-31 |           |
| Chromozym tPA | D-Phe-Gly-Arg      | 37.6                             | 117.6   | 3         |
| S-2288        | H-D-Ile-Pro-Arg    | 25.8                             | 304.2   | 12        |
| S-2366        | pyro-Glu-Pro-Arg   | 11.5                             | 267.8   | 23        |
| S-2238        | H-D-Phe-Pip-Arg    | 11.3                             | 86.6    | 8         |
| Chromozym X   | D-Nile-Gly-Arg     | 13.0                             | 48.6    | 4         |
| S-2302        | H-D-Pro-Phe-Arg    | 7.4                              | 38.3    | 5         |
| S-2765        | Z-D-Arg-Gly-Arg    | 13.3                             | 20.6    | 2         |
| Chromozym TRY | CBz-Val-Gly-Arg    | 5.6                              | 28.2    | 5         |
| S-2444        | pyro-Glu-Gly-Arg   | 1.5                              | 18.9    | 13        |
| S-2222        | Bz-Ile-Glu-Gly-Arg | 5.6                              | 16.4    | 3         |
| S-2403        | pyro-Glu-Phe-Lys   | 0.3                              | 5.9     | 19        |

【0032】

【発明の効果】このように本願発明により得られたFV

II 及び/または FV II a の改変体は、野生型の FV II に比べて明らかに高い酵素活性を有するものである。従って、本願発明の改変体は、血友病インヒター

患者への補充療法として極めて有効な薬剤となりうるものである。

【配列表】

SEQUENCE LISTING

<110> The Chemo-Sero-Therapeutic Research Institute

<120> Recombinant mutants of blood coagulation factor VII

<160> 12

<210> 1

<211> 1221

<212> DNA

<213> blood coagulation factor VII

<400> 1

|   |     |
|---|-----|
| GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG | 48  |
| Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu |     |
| 1 5 10 15   |     |
| TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG | 96  |
| Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys |     |
| 20 25 30  |     |
| GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC | 144 |
| Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp |     |
| 35 40 45  |     |
| CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG | 192 |
| Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln |     |
| 50 55 60  |     |
| CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC | 240 |
| Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn |     |
| 65 70 75 80   |     |
| TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC | 288 |
| Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly |     |
| 85 90 95  |     |
| GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT | 336 |
| Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys |     |
| 100 105 110   |     |
| CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA | 384 |
| Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr |     |
| 115 120 125   |     |
| CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA | 432 |
| Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg |     |
| 130 135 140   |     |
| AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC | 480 |
| Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro |     |
| 145 150 155 160   |     |
| AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG | 528 |
| Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln     |     |
| 165 170 175   |     |
| TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC | 576 |
| Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala |     |
| 180 185 190   |     |
| CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG | 624 |
| His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu |     |

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      195              200              205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG   672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
      210              215              220
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC   720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
      225              230              235              240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC   768
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
      245              250              255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG   816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
      260              265              270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG   864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
      275              280              285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG   912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
      290              295              300
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC   960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
      305              310              315              320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC   1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
      325              330              335
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC   1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
      340              345              350
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC   1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
      355              360              365
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC   1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
      370              375              380
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC   1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
      385              390              395              400
CTG CGA GCC CCA TTT CCC TAG   1221
Leu Arg Ala Pro Phe Pro
      405
<210> 2
<211> 406
<212> PRT
<213> blood coagulation factor VII
<400> 2
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1              5              10              15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
      20              25              30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp

```

35 40 45  
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln  
 50 55 60  
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn  
 65 70 75 80  
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly  
 85 90 95  
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys  
 100 105 110  
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr  
 115 120 125  
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg  
 130 135 140  
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro  
 145 150 155 160  
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln  
 165 170 175  
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala  
 180 185 190  
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
 195 200 205  
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg  
 210 215 220  
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn  
 225 230 235 240  
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
 245 250 255  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
 260 265 270  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
 275 280 285  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
 290 295 300  
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser  
 305 310 315 320  
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
 325 330 335  
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr  
 340 345 350  
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys  
 355 360 365  
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile  
 370 375 380  
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu  
 385 390 395 400  
 Leu Arg Ala Pro Phe Pro  
 405

&lt;210&gt; 3

&lt;211&gt; 1221

&lt;212&gt; DNA

<213> artificial sequence

<220>

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which both of the 159th Cysteine and the 164th Cysteine are replaced with Alanine, and cDNA sequence coding thereof.

<400> 3

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GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG      48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
   1           5           10          15
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG      96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
           20           25           30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC     144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
           35           40           45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG     192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
           50           55           60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC     240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
           65           70           75           80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC     288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
           85           90           95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT     336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
           100          105          110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA     384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
           115          120          125
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA     432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
           130          135          140
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC     480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro
           145          150          155          160
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG     528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
           165          170          175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC     576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
           180          185          190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG     624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
           195          200          205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG     672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
           210          215          220
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC     720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn

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225          230          235          240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC   768
His Asp Ile Ala Leu Arg Leu His Gln Pro Val Val Leu Thr Asp

          245          250          255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG   816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr

          260          265          270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG   864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu

          275          280          285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG   912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg

          290          295          300
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC   960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser

          305          310          315          320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC  1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser

          325          330          335
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC  1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr

          340          345          350
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC  1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys

          355          360          365
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC  1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile

          370          375          380
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC  1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu

          385          390          395          400
CTG CGA GCC CCA TTT CCC TAG                                     1221
Leu Arg Ala Pro Phe Pro

```

405

&lt;210&gt; 4

&lt;211&gt; 406

&lt;212&gt; PRT

&lt;213&gt; artificail sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which both of the 159th Cysteine and the 164th Cysteine are replaced with Alanine.

&lt;400&gt; 4

```

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1           5           10          15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
          20          25          30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
          35          40          45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln

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50                      55                      60  
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn  
 65                      70                      75                      80  
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly  
                     85                      90                      95  
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys  
                     100                      105                      110  
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr  
                     115                      120                      125  
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg  
                     130                      135                      140  
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro  
 145                      150                      155                      160  
 Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln  
                     165                      170                      175  
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala  
                     180                      185                      190  
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
                     195                      200                      205  
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg  
                     210                      215                      220  
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn  
 225                      230                      235                      240  
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
                     245                      250                      255  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
                     260                      265                      270  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
                     275                      280                      285  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
                     290                      295                      300  
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser  
 305                      310                      315                      320  
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
                     325                      330                      335  
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr  
                     340                      345                      350  
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys  
                     355                      360                      365  
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile  
                     370                      375                      380  
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu  
 385                      390                      395                      400  
 Leu Arg Ala Pro Phe Pro  
                     405

&lt;210&gt; 5

&lt;211&gt; 1221

&lt;212&gt; DNA

&lt;213&gt; artificial sequence

&lt;220&gt;



<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine, and cDNA sequence coding thereof.

<400> 5

```

GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG      48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
   1             5             10            15
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG      96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
           20           25           30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC     144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
       35           40           45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG     192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
       50           55           60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC     240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
       65           70           75           80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC     288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
           85           90           95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT     336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
       100          105          110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA     384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
       115          120          125
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA     432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
       130          135          140
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC     480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
       145          150          155          160
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG     528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
           165          170          175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC     576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
           180          185          190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG     624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
           195          200          205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG     672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
       210          215          220
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC     720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
       225          230          235          240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC     768

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His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
      245              250              255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG   816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
      260              265              270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG   864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
      275              280              285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG TGC CTC AAC GTG CCC CGG   912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg
      290              295              300
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC   960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
      305              310              315              320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC   1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
      325              330              335
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC   1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
      340              345              350
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC   1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
      355              360              365
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC   1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
      370              375              380
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC   1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
      385              390              395              400
CTG CGA GCC CCA TTT CCC TAG   1221
Leu Arg Ala Pro Phe Pro
      405

```

&lt;210&gt; 6

&lt;211&gt; 406

&lt;212&gt; PRT

&lt;213&gt; artificial sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine.

&lt;400&gt; 6

```

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1              5              10              15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
      20              25              30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
      35              40              45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
      50              55              60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn

```

65                      70                      75                      80  
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly  
                                  85                      90                      95  
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys  
                                  100                      105                      110  
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr  
                                  115                      120                      125  
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg  
                                  130                      135                      140  
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro  
 145                      150                      155                      160  
 Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln  
                                  165                      170                      175  
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala  
                                  180                      185                      190  
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
                                  195                      200                      205  
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg  
                                  210                      215                      220  
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn  
 225                      230                      235                      240  
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
                                  245                      250                      255  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
                                  260                      265                      270  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
                                  275                      280                      285  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg  
                                  290                      295                      300  
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser  
 305                      310                      315                      320  
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
                                  325                      330                      335  
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr  
                                  340                      345                      350  
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys  
                                  355                      360                      365  
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile  
                                  370                      375                      380  
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu  
 385                      390                      395                      400  
 Leu Arg Ala Pro Phe Pro  
                                  405

&lt;210&gt; 7

&lt;211&gt; 1221

&lt;212&gt; DNA

&lt;213&gt; artificial sequence

&lt;220&gt;

&lt;223&gt; Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding thereof.

<400> 7

|   |     |
|---|-----|
| GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG | 48  |
| Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu |     |
| 1 5 10 15   |     |
| TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG | 96  |
| Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys |     |
| 20 25 30  |     |
| GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC | 144 |
| Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp |     |
| 35 40 45  |     |
| CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG | 192 |
| Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln |     |
| 50 55 60  |     |
| CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC | 240 |
| Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn |     |
| 65 70 75 80   |     |
| TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC | 288 |
| Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly |     |
| 85 90 95  |     |
| GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT | 336 |
| Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys |     |
| 100 105 110   |     |
| CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA | 384 |
| Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr |     |
| 115 120 125   |     |
| CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA | 432 |
| Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg |     |
| 130 135 140   |     |
| AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC | 480 |
| Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro |     |
| 145 150 155 160   |     |
| AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG | 528 |
| Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln |     |
| 165 170 175   |     |
| TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC | 576 |
| Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala |     |
| 180 185 190   |     |
| CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG | 624 |
| His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu |     |
| 195 200 205   |     |
| GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG | 672 |
| Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg |     |
| 210 215 220   |     |
| GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC | 720 |
| Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn |     |
| 225 230 235 240   |     |
| CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC | 768 |

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
                     245                    250                    255  
 CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
                     260                    265                    270  
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
                     275                    280                    285  
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
                     290                    295                    300  
 CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960  
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser  
                     305                    310                    315                    320  
 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008  
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
                     325                    330                    335  
 AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC 1056  
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr  
                     340                    345                    350  
 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104  
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys  
                     355                    360                    365  
 GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152  
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile  
                     370                    375                    380  
 GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200  
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu  
                     385                    390                    395                    400  
 CTG CGA GCC CCA TTT CCC TAG 1221  
 Leu Arg Ala Pro Phe Pro

405

&lt;210&gt; 8

&lt;211&gt; 406

&lt;212&gt; PRT

&lt;213&gt; artificial sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu.

&lt;400&gt; 8

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu  
           1                    5                    10                    15  
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys  
                     20                    25                    30  
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp  
                     35                    40                    45  
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln  
                     50                    55                    60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn  
 65                      70                      75                      80  
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly  
                                  85                      90                      95  
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys  
                                  100                      105                      110  
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr  
                                  115                      120                      125  
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg  
                                  130                      135                      140  
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro  
 145                      150                      155                      160  
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln  
                                  165                      170                      175  
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala  
                                  180                      185                      190  
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
                                  195                      200                      205  
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg  
                                  210                      215                      220  
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn  
 225                      230                      235                      240  
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
                                  245                      250                      255  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
                                  260                      265                      270  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
                                  275                      280                      285  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
                                  290                      295                      300  
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser  
 305                      310                      315                      320  
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser  
                                  325                      330                      335  
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr  
                                  340                      345                      350  
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys  
                                  355                      360                      365  
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile  
                                  370                      375                      380  
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu  
 385                      390                      395                      400  
 Leu Arg Ala Pro Phe Pro  
                                  405

&lt;210&gt; 9

&lt;211&gt; 1206

&lt;212&gt; DNA

&lt;213&gt; artificial sequence

&lt;220&gt;

&lt;223&gt; Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA sequence coding thereof.

<400> 9

|   |     |
|---|-----|
| GCC AAC GCG TTC CTG GAG GAG CTG CCG CCG GGC TCC CTG GAG AGG GAG | 48  |
| Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu |     |
| 1 5 10 15   |     |
| TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG | 96  |
| Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys |     |
| 20 25 30  |     |
| GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC | 144 |
| Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp |     |
| 35 40 45  |     |
| CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG | 192 |
| Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln |     |
| 50 55 60  |     |
| CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC | 240 |
| Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn |     |
| 65 70 75 80   |     |
| TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC | 288 |
| Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly |     |
| 85 90 95  |     |
| GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT | 336 |
| Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys |     |
| 100 105 110   |     |
| CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA | 384 |
| Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr |     |
| 115 120 125   |     |
| CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA | 432 |
| Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg |     |
| 130 135 140   |     |
| AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC | 480 |
| Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro |     |
| 145 150 155 160   |     |
| AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG | 528 |
| Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln |     |
| 165 170 175   |     |
| TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC | 576 |
| Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala |     |
| 180 185 190   |     |
| CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG | 624 |
| His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu |     |
| 195 200 205   |     |
| GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG | 672 |
| Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg |     |
| 210 215 220   |     |
| GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC | 720 |
| Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn |     |
| 225 230 235 240   |     |
| CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC | 768 |

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
 245 250 255  
 CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
 260 265 270  
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
 275 280 285  
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
 290 295 300  
 CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960  
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu  
 305 310 315 320  
 TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG 1008  
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys  
 325 330 335  
 GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC 1056  
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr  
 340 345 350  
 CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC 1104  
 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His  
 355 360 365  
 TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152  
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys  
 370 375 380  
 CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT 1200  
 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe  
 385 390 395 400  
 CCC TAG 1206

Pro

&lt;210&gt; 10

&lt;211&gt; 401

&lt;212&gt; PRT

&lt;213&gt; artificial sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.

&lt;400&gt; 10

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu  
 1 5 10 15  
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys  
 20 25 30  
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp  
 35 40 45  
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln  
 50 55 60  
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn  
 65 70 75 80



Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly  
                                     85                                    90                                    95  
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys  
                                     100                                    105                                    110  
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr  
                                     115                                    120                                    125  
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg  
                                     130                                    135                                    140  
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro  
 145                                    150                                    155                                    160  
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln  
                                     165                                    170                                    175  
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala  
                                     180                                    185                                    190  
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu  
                                     195                                    200                                    205  
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg  
                                     210                                    215                                    220  
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn  
 225                                    230                                    235                                    240  
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp  
                                     245                                    250                                    255  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
                                     260                                    265                                    270  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
                                     275                                    280                                    285  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
                                     290                                    295                                    300  
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu  
 305                                    310                                    315                                    320  
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys  
                                     325                                    330                                    335  
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr  
                                     340                                    345                                    350  
 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His  
                                     355                                    360                                    365  
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys  
                                     370                                    375                                    380  
 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe  
 385                                    390                                    395                                    400  
 Pro

&lt;210&gt; 11

&lt;211&gt; 1206

&lt;212&gt; DNA

&lt;213&gt; artificial sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Gl

u-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA sequence coding thereof.

<400> 11

|   |     |
|---|-----|
| GCC AAC GCG TTC CTG GAG GAG CTG CCG CCG GGC TCC CTG GAG AGG GAG | 48  |
| Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu |     |
| 1 5 10 15   |     |
| TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG | 96  |
| Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys |     |
| 20 25 30  |     |
| GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC | 144 |
| Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp |     |
| 35 40 45  |     |
| CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG | 192 |
| Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln |     |
| 50 55 60  |     |
| CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC | 240 |
| Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn |     |
| 65 70 75 80   |     |
| TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC | 288 |
| Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly |     |
| 85 90 95  |     |
| GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT | 336 |
| Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys |     |
| 100 105 110   |     |
| CGG TGC CAC GAG GGC TAC TCT CTG CTG GCA GAC GGC GTG TCC TGC ACA | 384 |
| Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr |     |
| 115 120 125   |     |
| CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA | 432 |
| Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg |     |
| 130 135 140   |     |
| AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGC GGC AAG GTG TGC CCC | 480 |
| Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro |     |
| 145 150 155 160   |     |
| AAA GGC GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG | 528 |
| Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln |     |
| 165 170 175   |     |
| TTG TGT GGC GGC ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC | 576 |
| Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala |     |
| 180 185 190   |     |
| CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG | 624 |
| His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu |     |
| 195 200 205   |     |
| GGC GAG CAC GAC CTC AGC GAG CAC GAC GGC GAT GAG CAG AGC CGG CGG | 672 |
| Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg |     |
| 210 215 220   |     |
| GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC | 720 |
| Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn |     |
| 225 230 235 240   |     |
| CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC | 768 |
| His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp |     |
| 245 250 255   |     |

CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816  
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr  
 260 265 270  
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864  
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu  
 275 280 285  
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912  
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg  
 290 295 300  
 CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960  
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu  
 305 310 315 320  
 TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG 1008  
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys  
 325 330 335  
 GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC 1056  
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr  
 340 345 350  
 CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC 1104  
 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His  
 355 360 365  
 TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152  
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys  
 370 375 380  
 CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT 1200  
 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe  
 385 390 395 400  
 CCC TAG 1206

Pro

&lt;210&gt; 12

&lt;211&gt; 401

&lt;212&gt; PRT

&lt;213&gt; artificial sequence

&lt;220&gt;

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.

&lt;400&gt; 12

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu  
 1 5 10 15  
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys  
 20 25 30  
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp  
 35 40 45  
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln  
 50 55 60  
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn  
 65 70 75 80

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Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
      85              90              95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
      100             105             110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
      115             120             125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
      130             135             140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
      145             150             155             160
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
      165             170             175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
      180             185             190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
      195             200             205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
      210             215             220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
      225             230             235             240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
      245             250             255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
      260             265             270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
      275             280             285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
      290             295             300
Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
      305             310             315             320
Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
      325             330             335
Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
      340             345             350
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
      355             360             365
Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
      370             375             380
Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe
      385             390             395             400
Pro

```

【図面の簡単な説明】

【図 1】 F V I I の一次構造及び改変部位（星印）を示す図。

【図 2】 F V I I のプロテアーゼドメインアミノ酸配列を基にしたセリンプロテアーゼの基本構造を示す図。

【図 3】 X線立体構造既知の各種トリプシン族セリンプロテアーゼ間の 3D マルチアライメントを示す図。

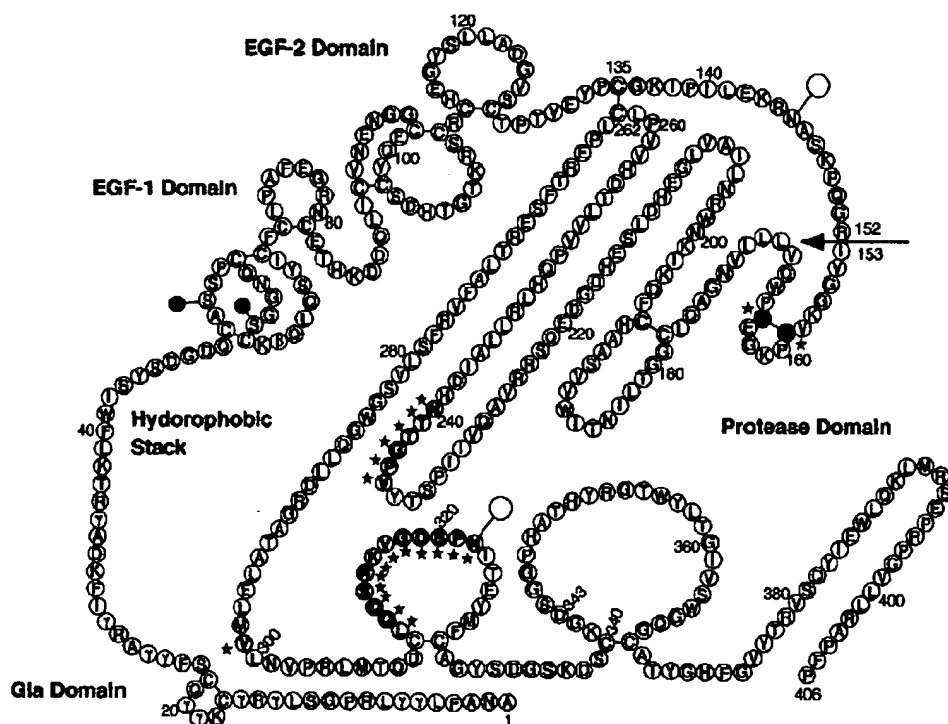
【図 4】 野生型 F V I I (F V I I - W i l d) 及び各種 F V I I 改変体のアミノ酸配列の一部を示す図。本

図は F V I I の 153 番目のインロイシンより C 末側のアミノ酸配列のみ示したもので、152 番目のアルギニンより N 末側のアミノ酸配列についてはいずれも改変は行っておらず野生型と同じである。

【図 5】 F V I I 改変体作製用プライマー配列を示す図。

【図 6】 F V I I 改変体発現ベクターの構築方法を示す図。

【图 1】



【図 2】

```

I V G G K V C P K G E C P W Q V L L L V N G A Q L C G G T L I N T I W V V S A A
153          βストランド1      βストランド2          βストランド3 192

H C F D K I K N W R N L I A V I G E H D L S E H D G D E Q S R R V A Q V I I P S
193          βストランド4          βストランド5          232

T Y V P G T T N H D I A L L R L H Q P V V L T D H V V P L C L P E R T F S E R T
233          βストランド6          272

L A F V R E S L V S G W G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q
273          βストランド7          βストランド8          312

Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T H Y
313          βストランド9          βストランド10 352

R G T W Y L T G I V S W G Q G C A T V G H F G V Y T R V S Q Y I E W L Q K L M R
353          βストランド11          βストランド12          392

S E P R P G V L L R A P F P
393

```

sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)  
sequence 2: ヒトトリプシン (pdb ID 1TRN)  
sequence 3: プタ血液凝固第IX因子 (pdb ID 1PFX)  
sequence 4: ウシトリプシン (pdb ID 1TLB)  
sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)  
sequence 6: ヒトプロテインC (pdb ID 1AUT)  
sequence 7: プタカリクレインA (pdb ID NPKA)  
sequence 8: ウシキモトリプシン (pdb ID 5CHA)  
sequence 9: プタエラスターゼ (pdb ID 3EST)  
sequence10: ヒト $\alpha$ トロンビン (pdb ID 1PPB)  
sequence11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)  
sequence12: ラットトニン (pdb ID 1TON)  
sequence13: ヒト好中球エラスターゼ (pdb ID 1HNE)  
sequence14: ヒトウロキナーゼ型プラスミノージェンアクチベータ (pdb ID 1LMW)  
sequence15: ヒトカテプシンG (pdb ID 1CGH)  
sequence16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)  
sequence17: ヒト組織型プラスミノージェンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのC<sub>α</sub>位置が1Å以内で保存されている構造保存部位を示す)

|              | βストランド5-βストランド6近傍の<br>アライメント       | βストランド8-βストランド9近傍の<br>アライメント          |
|--------------|------------------------------------|---------------------------------------|
|              | @@@@@@@@@:@@@:::.....@@@@@@@@@@@@  | @@@@@@@@@@@@@@@:.....@                |
| sequence 1:  | SRRVAQVIIPSTYVP---G-TTNHDIALLRLHQ  | ALEMLVNVPRMTQDCLQSKRVGDSNPITEYMFCA    |
| sequence 2:  | FINAAKIIRHPQYDR---K-TLNDIMLIKLS    | PDELQCLDAPVLSQAKCEA-S-Y---PGKITSNMFCV |
| sequence 3:  | RRNVIRAIPIHHSYNAT---VNKYSHDIALLEDE | ATILQYLKVLPIVDRATCL-R-ST-KFTIYSNMFC   |
| sequence 4:  | FISASKSIVHPSYNS---N-TLNDIMLIKLS    | PDVLKCLKAPILSDSSCRS-A-Y---PGQITSNMFC  |
| sequence 5:  | DHEVEVVIKHNRFT---E-TYDFDIAVLRLEK   | STRLKMLEVPPYDRNSCKL-S-S---SFIITQNMFC  |
| sequence 6:  | VLDIKEYFVHPVYSK---S-TTNDIDIALHLAQ  | TFVLNFQIKIPVVHNCESE-V-M---SNMVSNMLC   |
| sequence 7:  | FFGVYADFPHPGFNLSA-DGDKYSHDLMLLRQ   | PDEIQCVGLTLQHTNCA-D-AH-PDKVTESMLC     |
| sequence 8:  | KLKIAKVPKNSKYS---L-TINNDITLKLST    | PDRLQQASLPLLSNTNCKX-Y-W---GTKIKDAMIC  |
| sequence 9:  | YVGQKIVVHPYWT---D-DVAAGYDIALRLAQ   | AQTLQQAALPTVDYAISS-SSYW-GSTVENSVMC    |
| sequence 10: | ISMLEKIYIIPRYNW---RENLRDIALMKLKK   | PSVLQVNLPIVERPVCKD-S-T---RIRITDNMFC   |
| sequence 11: | HFSVAQVFLN-NYDA---E-NLNDILLIQLSS   | AQVLQELNVTYVT-FFC-----R-PHNICTF       |
| sequence 12: | RRLVRQSFRRHPDYP--LPVHDHSDNMLMLHLS  | SHDLQCVNIHLLSNEKCI-E-TY-KDNVTDVMLC    |
| sequence 13: | VFAQRFIFD-GYDP---V-NLNDIVILQLNG    | ASVLQELNVTYVT-SLC-----R-RSNVCTL       |
| sequence 14: | KFEVENLILHKDYSA-D-TLAHHNDIALKLRS   | PEQLKRVTVKLLISHREQQPH-YY-GSEVTTKMLC   |
| sequence 15: | HITARRAIRHPQYQNQ---R-TIQNDIMLLQLSR | TDTLTREVLQRVQDRQCLR-I-F---GSYDPRRQIC  |
| sequence 16: | KIKVEKQIHESYNS---V-PNLIDIMLLKLEK   | SYTLREVELRIMDEKACVD-Y-R---Y-YEYKFOVCV |
| sequence 17: | KFEVEKYIVHKEFDD---D-TYDNDIALLLKLS  | SERLKEAHVRLYPSSRCTSQH-LL-NRTVTDNMFC   |

## 【図4】

## VII-Wild

IVGGKVCPRGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDS PNITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

## VII-5

IVGGKVCAPRGEAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDS PNITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

## VII-6

IVGGKVCPRGECAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMCLNVPRLMTQDCLQQRKVGDS PNITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

## VII-30

IVGGKVCPRGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQRKVGDS PNITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

## VII-31

IVGGKVCPRGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

## VII-39

IVGGKVCPRGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHD  
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALRLHQPVVLT DHVVPLCLPERTFSERT  
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY  
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR  
SEPRPGVLLRAPFP

(下線部は改変部位を表す)

【図5】

①VII-PWN Sense ; 5'-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'

Factor VII Wild type のシグナル配列からのプライマーデザイン

5'-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'

Sali M V S Q A L R L L C L L

②VII-PWC AntiS ; 5'-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3'

Factor VII Wild type のカルボキシ末端までのプライマーデザイン

5'-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3'

BamHI

③VII-P5-1 Sense ; 5'-ATTGTGGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

④VII-P5-2 AntiS ; 5'-GACCTGCCATGGGGCCTCCCTTTGGGGGCCACCTTGCCCCCACAAT-3'

VII-5のプライマーデザイン (C159A, C164A)

5'-ATTGTGGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

3'-TAACACCCCCGTTCCACCGGGGTTTCCCCTCGGGGTACCGTCCAG-5'

I V G G K V A P K G E A P W Q V

⑤VII-P6-1 Sense ; 5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

⑥VII-P6-2 AntiS ; 5'-GACCTGCCATGGGGCCTCCCTTTGGGGCA-3'

VII-6のプライマーデザイン① (C164A)

5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

3'-ACGGGGTTTCCCTCCGGGTACCGTCCAG-5'

C P K G E A P W Q V

⑦VII-P6-3 Sense ; 5'-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3'

⑧VII-P6-4 AntiS ; 5'-CCGGGGCACGTTGAGGCACATGAGCTCCAG-3'

VII-6のプライマーデザイン② (V299C)

5'-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3'

3'-GACCTCGAGTACACGGAGTTCACGGGGCC-5'

L E L M C L N V P R

⑨VII-P30-1 Sense ; 5'-ATCCCCAGCAGTACGACAGGAAGACTCTGAACCAAGACATCGCGCTG-3'

⑩VII-P30-2 AntiS ; 5'-CAGCGCGATGTCGTGGTTTCAGAGTCTTCCTGTCGTACGTGCTGGGGAT-3'

VII-30のプライマーデザイン (VPGTTN→DRKTLN)

5'-ATCCCCAGCAGTACGACAGGAAGACTCTGAACCAAGACATCGCGCTG-3'

3'-TAGGGGTCGTGCATGCTGCTTCTGAGACTTGGTGCTGTAGCGCGAC-5'

I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense ; 5'-ATGACCCAGGACTGCGAAGCCTCCTACCTGGAAGATCACGGAGTACATG-3'

⑫VII-P31-2 AntiS ; 5'-CATGTACTCCGTGATCTTCCAGGGTAGGAGGCTTCGCAGTCTGGGTCAT-3'

VII-31のプライマーデザイン (LQSRKVGDSFN→EASYPGR)

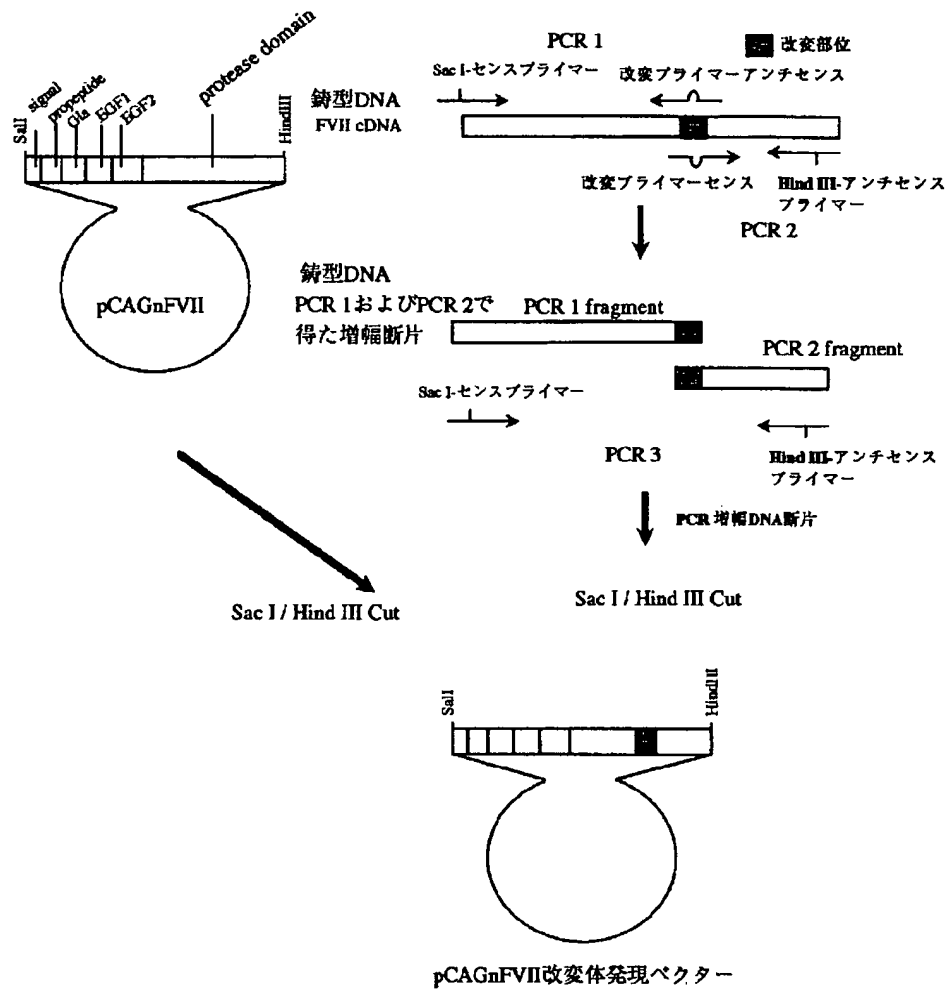
5'-ATGACCCAGGACTGCGAAGCCTCCTACCTGGAAGATCACGGAGTACATG-3'

3'-TACTGGGTCCTGACGCTTCGGAGGATGGGACCTTCTAGTGCCTCATGTAC-5'

M T Q D C E A S Y P G K I T E Y M



【図6】



フロントページの続き

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CA18 CA53 DC14 NA05 ZA532  
ZC542  
4H045 AA10 AA30 BA10 BA12 BA13  
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